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Induction of secretory pathway components in yeast is associated with increased stability of their mRNA

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The overexpression of certain membrane proteins is accompanied by a striking proliferation of intracellular membranes. One of the best characterized inducers of membrane proliferation is the 180-kD mammalian ribosome receptor (p180), whose expression in yeast results in increases in levels of mRNAs encoding proteins that function in the secretory pathway, and an elevation in the cell's ability to secrete proteins. In this study we demonstrate that neither the unfolded protein response nor increased transcription accounts for membrane proliferation or the observed increase in secretory pathway mRNAs. Rather, p180-induced up-regulation of certain secretory pathway

transcripts is due to a p180-mediated increase in the longevity of these mRNA species, as determined by measurements of transcriptional activity and specific mRNA turnover. Moreover, we show that the longevity of mRNA in general is substantially promoted through the process of its targeting to the membrane of the endoplasmic reticulum. With respect to the terminal differentiation of secretory tissues, results from this model system provide insights into how the expression of a single protein, p180, could result in substantial morphological and functional changes.

Introduction

A number of internal and external stimuli trigger the expansion of the ER membrane system and stimulate increases in levels of ER-localized proteins in both mammalian cells and in the yeast *Saccharomyces cerevisiae*. Particularly dramatic is the proliferation of membranes that occurs during the terminal differentiation of secretory cells. In the case of mammalian tissues with extremely high secretory capacity, such as pancreas or liver, the concentration of an integral ER ribosome receptor (p180) is especially high. Expression of p180 in yeast, a cell without a structural p180 homologue, leads to a proliferation of rough ER membranes accompanied by an increased secretory capacity (Wanker et al., 1995; Becker et al., 1999). Overexpression of numerous proteins, including endogenous yeast membrane proteins such as the HMG CoA reductase isoforms (Hmg1p and Hmg2p) or the peroxisomal protein Pex15p, results in the proliferation of smooth membranes (Wright et al., 1988; Parrish et al., 1995; Koning et al.,

1996; Elgersma et al., 1997). Membrane proliferation and elevated levels of ER-localized chaperones are also associated with expression of certain forms of cytochrome P450 (Menzel et al., 1996, 1997). Only in the case of p180 expression, however, have increases in rough membranes as well as secretory capacity been reported (Becker et al., 1999).

The proliferation of certain ER-specific proteins results from disturbances within the lumen of the ER sensed via a well-characterized mechanism known as the unfolded protein response (UPR).^{*} The UPR represents a signal transduction pathway in yeast that links stress in the ER to transcription of genes encoding ER-resident proteins (Patil and Walter, 2001; Spear and Ng, 2001). Several similarities exist between induction of the UPR and proliferation of ER membranes upon expression of p180. In both processes, the levels of mRNAs that encode ER-localized chaperones and phospholipid biosynthetic genes are elevated (Cox et al., 1997; Becker et al., 1999; Block-Alper et al., 2002). Furthermore, p180-induced membrane proliferation is accompanied by increased levels of secretory pathway mRNAs, another feature of cells undergoing a UPR (Becker et al., 1999; Travers et

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Key words: secretion; endoplasmic reticulum; yeast; mRNA; membrane biogenesis

^{*}Abbreviations used in this paper: FP, free polysomes; GFP, green fluorescent protein; MBP, membrane-bound polysomes; UPR, unfolded protein response.

al., 2000). Increased secretory capacity, a prominent feature of p180 expression, has not been reported as a consequence of the UPR.

Cells that are deleted for *IRE1* are unable to undergo a UPR. Through analysis of such mutants, the UPR has been linked to membrane-related cellular processes such as phospholipid synthesis, protein modification and secretion, and ER-associated protein degradation (Cox et al., 1997; Friedlander et al., 2000; Travers et al., 2000). These mutational analyses, coupled with the observation that levels of ER-localized proteins were elevated in the UPR, led us to investigate the relevance of the UPR to membrane proliferation stimulated by p180 expression in yeast. We conclusively demonstrate in this study that p180-induced membrane proliferation, as well as increasing mRNA levels of ER-localized proteins and phospholipid biosynthetic enzymes, occurs by an *Ire1p-independent* mechanism.

Instead, we report here the involvement of a novel mechanism that potentially regulates the increased abundance of secretory pathway component transcripts observed during p180-induced membrane proliferation. Our results show that p180 expression stabilizes secretory component mRNAs, and that targeting of any mRNA to the ER membrane is

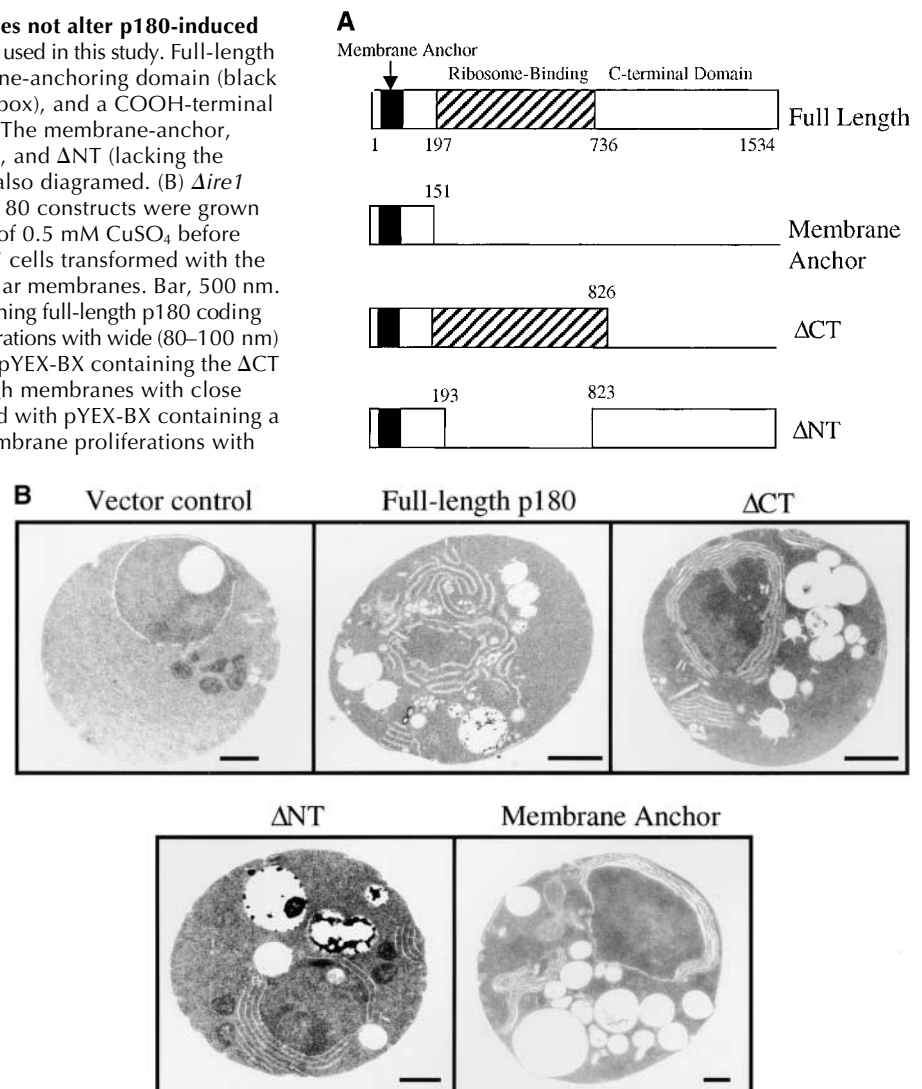
capable of mediating a substantial increase in its longevity. The conclusions we draw from our work in this model system could provide significant insights into processes occurring during the terminal differentiation of mammalian secretory tissues.

Results

P180-induced membrane biogenesis and elevated levels of secretory and lipid biosynthetic mRNAs occurs independently of the unfolded protein response

To determine if *IRE1* is required for p180-induced membrane proliferation, a strain was created in which signaling through the UPR was disabled by replacing the entire coding region of *IRE1* with the *HIS3* gene. This $\Delta ire1$ strain was found to be hypersensitive to agents that lead to the accumulation of unfolded proteins in the ER, such as treatment with the glycosylation inhibitor tunicamycin (unpublished data). As shown previously (Cox et al., 1997), these $\Delta ire1$ cells were unable to induce transcription of genes such as *KAR2* and *INO1* that are known to increase during treatment with tunicamycin (see Fig. 2, compare lanes 3 and 6).

Figure 1. Disruption of the *IRE1* gene does not alter p180-induced membrane proliferations. (A) P180 constructs used in this study. Full-length p180 contains an amino-terminal membrane-anchoring domain (black bar), a ribosome-binding domain (hashed box), and a COOH-terminal domain of unknown function (white box). The membrane-anchor, ΔCT (lacking the COOH-terminal domain), and ΔNT (lacking the ribosome-binding domain) constructs are also diagrammed. (B) $\Delta ire1$ cells transformed with vector or various p180 constructs were grown for 5 h in selective media in the presence of 0.5 mM CuSO_4 before preparation for electron microscopy. $\Delta ire1$ cells transformed with the empty pYEX-BX vector have few intracellular membranes. Bar, 500 nm. $\Delta ire1$ cells transformed with pYEX-BX containing full-length p180 coding sequence give rise to rough membrane proliferations with wide (80–100 nm) spacing. Bar, 1 μm . $\Delta ire1$ cells expressing pYEX-BX containing the ΔCT construct result in the proliferation of rough membranes with close spacing. Bar, 1 μm . $\Delta ire1$ cells transformed with pYEX-BX containing a sequence encoding ΔNT have smooth membrane proliferations with wide (80–100 nm) spacing. Bar, 500 nm. $\Delta ire1$ cells transformed with pYEX-BX containing the membrane anchoring region only have smooth membrane proliferations with close spacing. Bar, 500 nm.



We first asked if the hallmark of p180 expression, proliferation of ER membranes, is affected in an *Δire1* strain. Various p180 constructs (Fig. 1 A) were expressed in the *Δire1* strain from the high copy pYEX-BX plasmid under the control of the strong, inducible *CUP1* promoter. Proliferation of intracellular membranes was examined by electron microscopy. The micrographs indicate that the status of the *IRE1* gene had no bearing on membrane proliferation in p180-expressing cells, as expression of the various p180 constructs in *Δire1* cells induced membranes with the same morphologies as in wild-type cells (Fig. 1 B). All constructs induced some form of membrane, either perinuclear or membranes located in the periphery of cells, or both. Full-length p180 and Δ NT induced membranes with the characteristic 80–100 nm spacing (Becker et al., 1999), whereas the Δ CT and MA constructs, which lack the COOH-terminal domain, induced membranes with much closer spacing. These results suggest p180-induced membrane proliferation may occur in a UPR-independent fashion.

In all cases where it was examined, *IRE1* was required for the up-regulation of chaperones such as Kar2p. We were therefore eager to ascertain the requirement of UPR signaling in the p180-induced up-regulation of mRNAs encoding secretory pathway and phospholipid biosynthetic genes, including *KAR2*. The steady-state levels of these mRNAs in the *Δire1* strain were examined. Of the p180 constructs characterized previously (Wanker et al., 1995; Becker et al., 1999), the Δ CT construct, comprised of the membrane-spanning and ribosome-binding regions (Fig. 2 A), produced the largest increase in these mRNAs. Accordingly, Δ CT was used to study the relationship between this aspect of the p180 phenotype and the UPR. RNA was isolated for Northern blot analysis from wild-type and *Δire1* cells harboring the empty pYEX-BX vector in the presence or absence of tunicamycin and yeast expressing the Δ CT form of p180. Genes involved

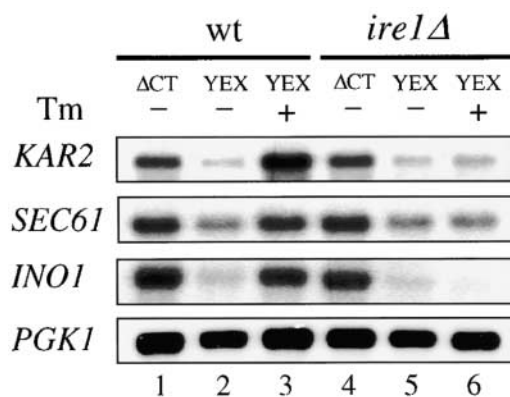


Figure 2. Increases in the steady-state levels secretory pathway mRNAs upon p180 induction occur independently of *IRE1*. Northern blot analysis was performed on RNA isolated from wild-type yeast and yeast in which the *IRE1* gene had been disrupted. Yeast cells were transformed with the pYEX-BX vector or with pYEX-BX containing the coding sequence for a COOH-terminal deletion of p180 (Δ CT). Strains were grown for 6 h in selective media in the presence of 0.5 mM CuSO_4 . Tunicamycin (Tm) was added to one vector-control culture to 2 $\mu\text{g}/\text{ml}$ for 2 h before harvesting of cells and isolation of RNA. Northern blot analyses are shown for *SEC61*, *KAR2*, and *INO1* mRNA.

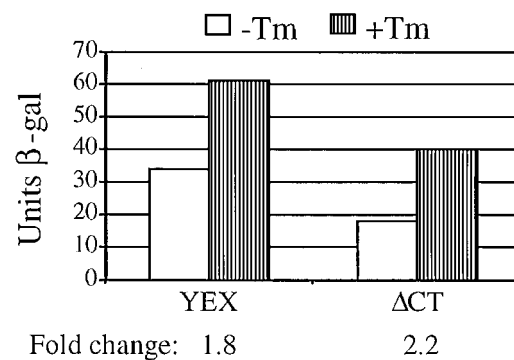


Figure 3. Expression of Δ CT does not increase activity at the UPRE. β -galactosidase activity was measured in extracts of BY4733 cells harboring the UPRE-LacZ plasmid, pMCZ-Y, and pYEX-BX or pYEX-BX containing Δ CT. Cells were grown for 5 h in selective media with 0.5 mM CuSO_4 in the presence or absence of tunicamycin (+Tm).

in translocation (*SEC61*), protein folding in the ER (*KAR2*) and inositol phospholipid biosynthesis (*INO1*) were compared for each strain. A housekeeping gene, phosphoglycerate kinase (*PGK1*), was used as a control. Expression of Δ CT in both wild-type and *Δire1* strains induced comparable steady-state mRNA levels of *KAR2*, *SEC61*, and *INO1* (Fig. 2, compare lanes 1 and 2 with lanes 4 and 5). Deletion of *IRE1*, however, abolished up-regulation of these mRNAs in response to tunicamycin. These results are consistent with the supposition that the cellular response to p180 induction is very likely UPR-independent.

Genes whose transcription is induced by the UPR contain a 22-base pair UPR responsive element (UPRE) in their upstream regulatory regions (Mori et al., 1992; Kohno et al., 1993). To confirm that the expression of p180 does not induce the UPR, assays measuring activity at this 22-base pair UPRE were performed. The plasmid pMCZ-Y, which encodes a fusion between the UPRE of the *KAR2* gene, minimal *CYC1* promoter elements and the coding sequence for the *Escherichia coli* β -galactosidase enzyme, has been used previously for such assays (Mori et al., 1996). As expected in cells harboring pMCZ-Y, increased β -galactosidase activity was seen as a result of tunicamycin treatment. In extracts from cells transformed with the pYEX-BX vector and pMCZ-Y, β -galactosidase activity increased approximately twofold after 2 h treatment with 2 $\mu\text{g}/\text{ml}$ tunicamycin (Fig. 3). In contrast, expression of Δ CT in pMCZ-Y-harboring cells slightly lowered β -galactosidase activity. Δ CT-expressing cells did not show a compromised UPR, as β -galactosidase activity was increased approximately twofold in extracts from tunicamycin-treated Δ CT cells compared with untreated Δ CT cells. Together, these data provide conclusive evidence that expression of Δ CT does not induce the unfolded protein response, nor is the UPR required for the p180-associated increases in levels of secretory pathway mRNA or intracellular membrane proliferation.

Transcriptional regulation is not responsible for increased levels of certain secretory pathway mRNAs in p180-expressing cells

An alternative mechanism governing the p180-induced increase in levels of mRNAs encoding secretory pathway pro-

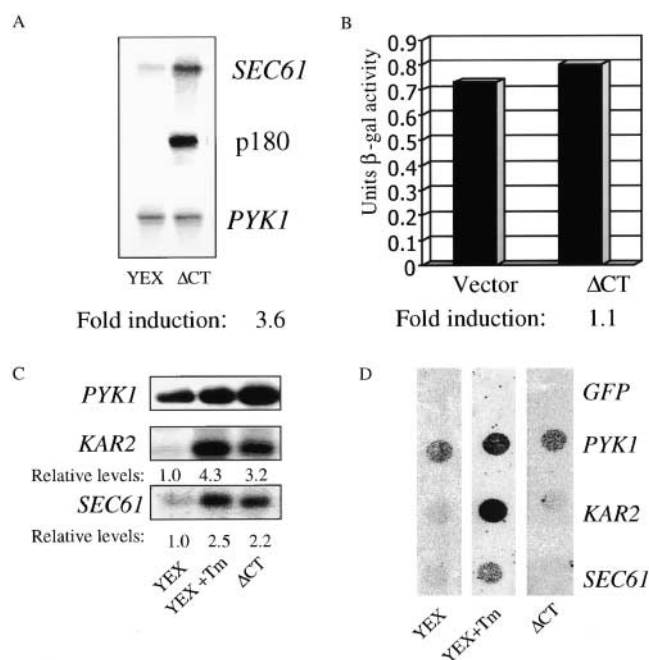


Figure 4. An increased level of *SEC61* mRNA is not regulated transcriptionally upon p180 induction. Effect of Δ CT expression on *SEC61* promoter activity was measured in extracts of SEY6210 cells harboring a *SEC61-LacZ* construct in pRS314 as well as a pYEX-BX vector or Δ CT-containing pYEX-BX plasmid. Cells were grown for 5 h in selective media in the presence of 0.5 mM CuSO_4 before preparation of extracts for assays. (A) Northern blot of RNA isolated from cultures used for the β -galactosidase assay. (B) β -galactosidase assay. (C and D) Northern blot analysis and transcription run-on assays, respectively, of BY4733 cells transformed with pYEX-BX vector, pYEX-BX vector + tunicamycin (Tm), or pYEX-BX containing Δ CT. Transcription of *KAR2* and *SEC61* was induced in the vector-only strain by the addition of Tm to 2 $\mu\text{g/ml}$ for 2 h before the harvesting of cells for preparation of RNA. Transcription and steady-state levels of *SEC61* and *KAR2* mRNA were compared with those of the *PYK1* gene. GFP was used as a control for nonspecific binding in the run-on assay.

teins might be the activation of a second signal transduction pathway, analogous to the UPR. Such a pathway would link the endoplasmic reticulum and the nucleus and result in increased transcription of genes encoding secretory pathway proteins. To determine if such a pathway exists, fusions were created between the promoter of a p180-induced gene (*SEC61*) and the *E. coli* LacZ gene. Levels of the *SEC61* mRNA show significant increases due to the expression of p180 (Fig. 4 A), and if these increases are due to transcriptional induction, increased activity at the *SEC61* promoter should be measurable by increases in activity of the β -galactosidase enzyme. Surprisingly, no significant difference was observed in β -galactosidase activity between vector control cells and those expressing Δ CT (Fig. 4 B). (Although unlikely, we cannot formally exclude the possibility that the *SEC61-LacZ* construct does not contain crucial elements of the *SEC61* promoter, should these lie outside the 1,000-base pair segment 5' to the *SEC61* coding region that we fused to LacZ.) A testable alternative hypothesis is that the changes in the level of the *SEC61* mRNA, and presumably other secretory pathway mRNAs in p180-expressing cells,

occur not because of increased transcription; rather, the changes reflect decreases in mRNA degradation.

To assess the actual transcription levels of the *SEC61* and *KAR2* genes, we used a transcription run-on assay. Transcription of these genes was normalized against transcription of the pyruvate kinase (*PYK1*) gene, whose mRNA levels remain unchanged in the presence of p180. The gene encoding green fluorescent protein (GFP), which is not present in yeast, was used as a control for nonspecific binding of radio-labeled transcripts. Northern analysis showed that expression of Δ CT resulted in an increase in the steady-state levels of the *SEC61* and *KAR2* mRNAs similar to those caused by induction of the UPR (YEX + Tm cells) (Fig. 4 C). However, increased transcription of these genes was not observed in the run-on assay (Fig. 4 D), in contrast to tunicamycin-treated cells. These data show that increases in the steady-state levels of *KAR2* and *SEC61* mRNA caused by p180 expression are not due to transcriptional activity. We propose that a decrease in mRNA turnover is responsible.

P180 stabilizes mRNAs that are targeted to the secretory pathway

To directly measure the turnover of the *SEC61* mRNA, transcription of the endogenous *SEC61* gene was placed under the control of a tetracycline-repressible promoter, creating a means to halt transcription and monitor decay of this mRNA species. The entire DNA sequence between the start codon of the upstream divergently transcribed *CSR1* gene and the *SEC61* start codon was replaced with elements allowing control of *SEC61* expression by tetracycline. This *tet-SEC61* strain produced $\sim 90\%$ of wild-type levels of *SEC61* mRNA in the absence of added drug (unpublished data). Upon addition of the tetracycline analogue, doxycycline, transcription of the *SEC61* gene ceases. Decay of the *SEC61* mRNA was monitored by quantitative Northern blotting in a time-course after drug addition in cells expressing the Δ CT construct and vector control cells. *PGK1* mRNA is a housekeeping gene whose expression is not affected by p180 and was thus used as a control (LaGrandeur and Parker, 1999). In vector control cells, the half-life of *SEC61* mRNA was ~ 5 min, whereas in Δ CT-expressing cells, nearly all of the *SEC61* mRNA remained after 40 min (Fig. 5 A). This value may be conservative, as one of our experiments indicated the half-life for *SEC61* mRNA in Δ CT-expressing cells was in excess of 100 min (unpublished data). To verify that ribosome binding activity was integral to mRNA stabilization, we performed the same experiments in strains expressing full-length p180, which has ribosome binding activity, and in two strains that do not, namely Membrane Anchor and Δ NT (Fig. 1 A), as well as in Δ CT and vector-only strains. As can be seen in Fig. 5 B, both Δ CT and full-length p180-expressing strains showed a significant prolongation of *SEC61* mRNA half-life ($t_{1/2} > 45$ min in both cases), whereas strains expressing constructs that do not bind ribosomes were essentially similar to controls ($t_{1/2}$ for Δ NT, 6 min; Membrane Anchor, 7 min; and vector only, 5 min). Together, these data demonstrate a profound increase in the stability of *SEC61* mRNA in p180 and Δ CT-expressing yeast.

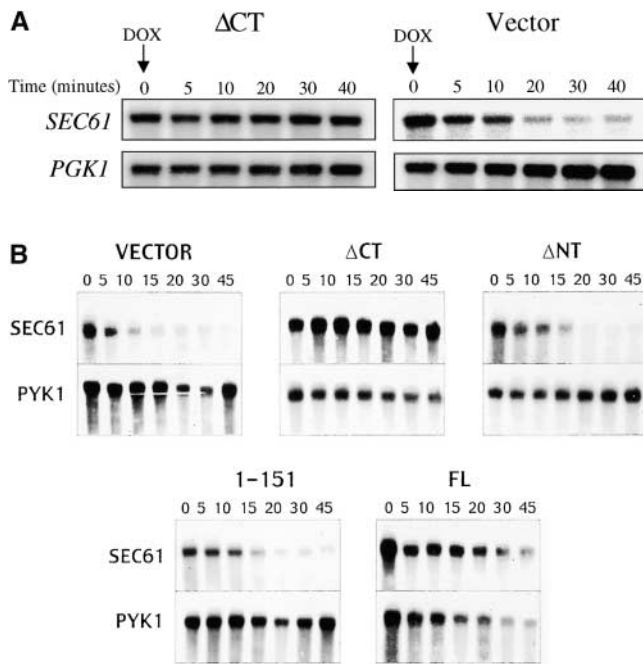


Figure 5. The half-life of *SEC61* mRNA is increased in cells expressing p180. (A) Northern blot of total cellular RNA extracted from BY4733 cells with a tetracycline-repressible promoter integrated at the *SEC61* locus following doxycycline arrest of transcription. Strains were transformed with pYEX-BX (vector) or pYEX-BX containing Δ CT and grown to mid-log phase in the presence of 0.5 mM CuSO_4 before the addition of doxycycline to 5 $\mu\text{g}/\text{ml}$. Cells were harvested at the indicated times and RNA isolated for analysis. Levels of *SEC61* mRNA were normalized to the *PGK1* mRNA. Four independent time-course analyses were performed, and the resulting half-life represents an average of the four experiments. DOX, doxycycline. (B) Same as in A, only examining *SEC61* mRNA levels in the other p180 expressing strains (diagrammed in Fig. 1 A). Values for half-lives, based on two separate experiments, are given in the text.

Since a number of secretory pathway mRNAs (which are translated on the endoplasmic reticulum) are up-regulated by expression of p180 (Becker et al., 1999), one hypothesis is that targeting these mRNAs to the endoplasmic reticulum is sufficient to cause a decrease in their turnover in a manner dependent on the ER-localized p180. The turnover rate of constructs that differ only in the presence of an ER-targeting signal could be used to determine if ER-targeting is sufficient to reduce the rate of decay of an mRNA that is normally localized to the cytoplasm. Two constructs were generated: one that encodes GFP, and another that encodes a fusion of the KAR2 signal sequence (KAR2ss) to GFP, yielding KAR2ss-GFP. These constructs were generated in pCM182 (Gari et al., 1997), such that transcription of these constructs is under the control of a tetracycline-repressible promoter. Rates of decay of the GFP and KAR2ss-GFP mRNA were compared in vector control and Δ CT-expressing cells. KAR2ss-GFP mRNA had a half-life of 6 min in the vector control strains, and a half-life of 27.5 min in Δ CT-expressing cells, indicating ~ 4.5 -fold decrease in the decay rate of the mRNA in Δ CT-expressing cells (Fig. 6). The GFP mRNA, had a half-life of 5.4 min and 12 min in vector control and Δ CT-expressing cells, respectively, only slightly more than a twofold difference in half-life for

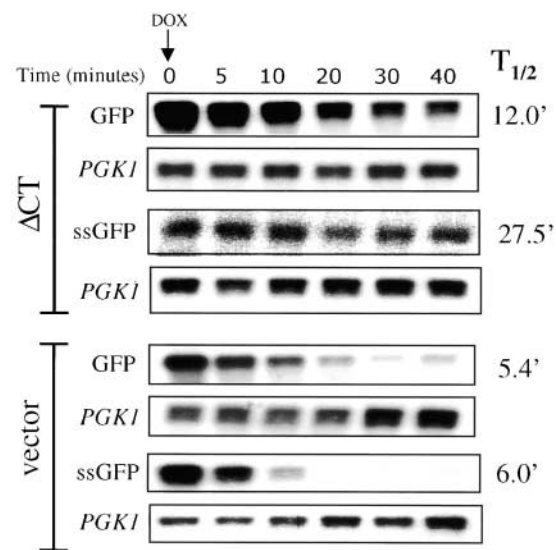


Figure 6. The half-life of GFP harboring the KAR2 signal sequence is increased in cells expressing p180. Northern blot of total cellular RNA extracted from BY4733 cells transformed with tetracycline-repressible constructs encoding GFP or a KAR2 signal sequence GFP (KAR2ss-GFP). Decay rates were compared for strains harboring the pYEX-BX vector or pYEX-BX containing Δ CT. Strains were grown to mid-log phase in the presence of 0.5 mM CuSO_4 before the addition of doxycycline to 5 mg/ml. Levels of GFP mRNAs were normalized to the *PGK1* mRNA. Four independent time-course analyses were performed, and the resulting half-life represents an average of the four experiments. DOX, doxycycline.

nontargeted GFP mRNA in Δ CT-expressing cells. These results suggest that Δ CT preferentially stabilizes ER-targeted mRNA species.

The fact that the half-life of the ER-targeted GFP mRNA increased substantially led us to investigate whether mRNAs exhibiting increased longevity are indeed physically associated with the site of p180 localization, the rough ER. Accordingly, we undertook an analysis of mRNA distribution on membrane-bound and free polysomes derived from vector control and Δ CT-expressing cells. RNA was isolated from free polysomes (FP) and membrane-bound polysomes (MBP) by taking advantage of the different sedimentation properties of membrane and soluble fractions (see Materials and methods). Probes were generated from fragments of *KAR2* and *SEC61*, as well as from the coding regions of *PEP4* and *PGK1*. *PEP4*, which encodes a vacuolar protease, is targeted to the secretory pathway and is used as a marker for MBP-specific mRNA. *PGK1* mRNA encodes the soluble phosphoglycerate kinase that is present primarily in FP-containing fractions. The histograms in Fig. 7, derived from quantitative Northern blotting, demonstrate that the increased amounts of *KAR2* and *SEC61* mRNAs in Δ CT-expressing cells are almost exclusively associated with their presence in polysomes derived from the membrane fraction. As expected, *PEP4* mRNA was also determined to be primarily associated with the MBP fraction, and increased slightly upon Δ CT expression. Interestingly, the p180-unresponsive *PGK1* mRNA that was present primarily in the FP fraction of vector-expressing cells (as expected), showed a slight shift in its distribution toward the MBP fraction in Δ CT-express-

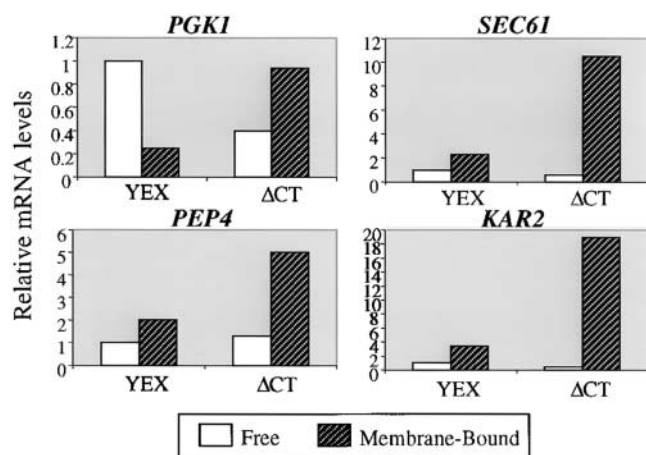


Figure 7. Messenger RNAs that encode components of the secretory pathway are highly associated with membrane-bound polysomes in p180-expressing cells. SEY6210 cells transformed with the pYEX-BX (YEX) vector or YEX containing the Δ CT form of p180 were induced with 0.5 mM CuSO_4 for 6 h. Free and membrane polysomes were isolated by differential centrifugation. RNA was isolated as described in Materials and methods and probed for *PGK1*, *PEP4*, *SEC61*, and *KAR2*. The graphs for each gene represent relative mRNA levels where the mRNA from the free polysome fraction of YEX was set to 1. White bars, mRNA from free polysomes; striped bars, mRNA from membrane-bound polysomes.

ing cells. This may be a consequence of the overall increase in membrane-bound ribosomes in Δ CT-expressing cells that we observed previously (Wanker et al., 1995).

This yeast model reproduces many of the events associated with the acquisition of a high secretory capacity, and may reflect a conserved mechanism that occurs during cellular differentiation in other organisms. It will thus be interesting to determine whether p180 plays a similar role in the terminal differentiation of secretory cells in higher eukaryotes.

Discussion

The work presented here describes a novel, and UPR-independent, means for increasing ER components featuring a p180-mediated stabilization of mRNAs on the ER membrane. This finding sets the stage for future studies into the actual molecular dynamics of such a process. Deletion of *IRE1*, which encodes an essential signaling molecule of the UPR, hindered neither the proliferation of p180-inducible membranes nor the up-regulation of mRNAs encoding various secretory pathway and phospholipid biosynthetic markers. We demonstrated that the increased level of *SEC61* mRNA in p180-expressing cells was not regulated at the transcriptional level, but rather at the level of mRNA turnover. Modification of an mRNA (that is normally translated in the cytosol) to enable its targeting to the ER caused an increase in its stability in the presence of p180. Membrane fractionation demonstrated that an increased stability of mRNAs targeted to the secretory pathway could be correlated with their increased association with the ER membrane in p180-expressing cells.

In contrast to the induction of ER chaperones such as *KAR2* in response to ER stress, the requirement for *IRE1* for

membrane proliferation is a subject of some debate. One group observed that *IRE1* was essential for *KAR2* mRNA induction in response to cytochrome p450 overexpression; however, it was not necessary for the production of ER-like membranes that arise upon expression of the protein (Menzel et al., 1997). A subsequent report demonstrated that *IRE1* was essential for the proliferation of cytochrome p450-induced membranes, and revealed that differences in strain background accounted for the conflicting results (Takewaka et al., 1999). In our experiments, the *IRE1* gene was deleted in two nonisogenic strains and found to be dispensable for p180-induced membrane proliferation and elevated levels of *KAR2* (unpublished data).

Our results also address the role of Ire1p in the inositol response and in membrane biogenesis. Phosphatidylinositol is one of the major lipids of membranes in eukaryotic cells. An understanding of any type of membrane proliferation will depend on an elucidation of how the need for additional membrane lipid is met. There appear to be multiple mechanisms by which a key element in this lipid's metabolism, the *INO1* gene, is regulated. It has been shown that deletion of *IRE1* results in inositol auxotrophy, suggesting that the UPR and phospholipid biosynthesis may be linked (Nikawa and Yamashita, 1992). Moreover, wild-type cells that were induced to undergo a UPR had increased *INO1* transcription and deletion of *OPI1*, a gene that encodes an inhibitor of inositol phospholipid synthesis restored inositol prototrophy to the Δ ire1 strain (Cox et al., 1997).

On the other hand, we demonstrate here that *INO1* mRNA can be up-regulated in the presence of p180 in a UPR-independent fashion. Similarly, Stroobants et al. (1999) showed that the inositol response is not necessarily linked to the UPR, as *ire1Δ* cells grown in the presence of oleate as the sole carbon source were capable of undergoing membrane proliferation upon overexpression of Hmg1p and the peroxisomal membrane protein, Pex15p (Stroobants et al., 1999). Others had shown that overexpression of HMG CoA-reductase (Hmg1p), which triggers the proliferation of karmellae, impaired the growth of *ire1Δ* cells, suggesting a block in membrane biogenesis, although membrane biogenesis per se was not assessed (Cox et al., 1997). They demonstrated that the level of *INO1* mRNA was activated in response to growth on oleate, dependent on the presence of oleate-specific transcriptional activators. The observation that there may be multiple mechanisms for activating *INO1* is further substantiated by our observation that Ino2p, a basic helix-loop-helix transcription factor essential for the production of *INO1* mRNA, is essential for the formation of p180-induced membranes (Block-Alper et al., 2002). How *INO2* is activated in response to p180 expression is unknown; however, it likely plays a critical role in the regulation of several phospholipid biosynthetic genes involved in the proliferation of intracellular membranes. Thus, it appears that the product of the *INO1* gene, inositol-1-phosphate, is an important phospholipid component that is likely activated by a variety of mechanisms, Ire1p-dependent and -independent, during conditions of ER stress.

Microsomes isolated from yeast cells expressing p180 constructs that contain the ribosome binding domain (p180-FL and Δ CT) were shown to bind 2–4 times as many ribosomes

as control microsomes in an in vitro ribosome-binding assay (Wanker et al., 1995). The high affinity of p180 for ribosomes ($K_d = 10\text{--}20\text{ nM}$) could account for the presence on the ER membrane of some “free polysomes” containing the normally cytosolically translated mRNAs (such as *PGK1*) (Fig. 7). Importantly, the overall level of *PGK1* mRNA between p180-expressing and control cells remained unchanged. This suggests that mere recruitment of polysomes to the ER is not solely responsible for mRNA stabilization and that other factors are likely involved. Such factors could include targeting coupled with translation of mRNAs and/or protein translocation on the ER membrane.

Targeting of mRNA to the membrane in the presence of p180 influences its turnover. This was shown in experiments using tetracycline-repressible forms of GFP and KAR2 signal sequence–modified GFP (Fig. 6) where mRNA turnover decreased about two- to threefold. Another contributing factor would be translation on the membrane. This is supported by our data on *SEC61* mRNA presented in Fig. 5, where turnover rates decreased by at least 8-fold and in other experiments by as much as 10- or 20-fold. Sec61p is a very hydrophobic pore-forming protein with multiple membrane-spanning domains. A previous study using a biological assay supports at least one of our interpretations. The investigators observed that the targeting the mRNA of a cytosolically translated histone bearing a hydrophobic signal peptide prevented its otherwise rapid cell cycle–dependent degradation (Zambetti et al., 1990). This mislocalization of mRNAs to the ER presumably prevented their degradation by cytosolic enzymes.

In p180-expressing cells, membrane-localized mRNAs could achieve protection from degradation by their physical association with ribosomes that are tightly bound to the membrane. Another interesting possibility is that mRNAs that are targeted to the ER interact directly with p180. If such an interaction occurs, it may be with regions traditionally recognized to promote mRNA stability, such as the 3'UTR. Interestingly, Lande et al. (1975) reported that in WI-38 cells (a human fibroblast cell line with well-developed rough ER) mRNAs can remain associated with the ER membrane after the membranes have been stripped of ribosomes (Lande et al., 1975). The poly-A portion of these mRNAs remains attached even following extensive treatment with RNase. An interesting and testable hypothesis is that p180 binds elements at or near the poly-A tail of certain mRNAs, thereby increasing their association with the membrane and protecting them from degradation. Lastly, p180 could be associating with both forms of RNA, ribosomal and messenger, stabilizing the translational complex. In this way an mRNA could experience increased levels of translation, surrounded by a variety of factors that render it inaccessible to enzymes involved in its turnover.

We have recently shown that Ino2p is required for generalized membrane proliferation (Block-Alper et al., 2002). Its deletion yields strains incapable of producing membranes of any kind in response to overexpression of membrane proteins, including all forms of p180 (see Fig. 1), and Hmg1p. Yet in this study, only ribosome-binding forms of p180 were able to stabilize secretory pathway mRNAs. This suggests that at least two steps are required for the production of

functional ER in yeast. The first requires an intact *INO2* gene and is needed for proliferation of the lipid bilayer common to all proliferated membranes. The second mechanism, involving p180 incorporation into the bilayer, enables higher expression of key proteins that will be integrated into the organelles of the secretory pathway, rough ER being a prime example. Thus, overexpression of any one of a variety of membrane proteins will trigger membrane biogenesis, as has been observed by a number of groups. When the inducer contains a ribosome binding domain, the longevity of key mRNAs will be enhanced, leading to the production of membranes containing the appropriate machinery to participate in the secretory pathway. This is precisely what is seen in the case of the Δ CT construct, which is little more than a membrane anchor with a ribosome binding domain. The membrane anchor confers the protein's ability to stimulate *INO2*-dependent bilayer formation, while the ribosome binding domain provides the most efficient stabilizer of specific mRNAs.

Thus far, these experiments do not address the role of p180 in mammalian secretory cells. It is still unknown whether the events that occur upon expression of a foreign membrane protein in yeast reproduce those that occur during terminal differentiation of cells with a high capacity for secretion. Our results, however, are promising. As the complexity and functionality of the membranes produced in response to p180 expression far exceeds that which is seen for other proteins, our studies assume a greater significance. The fact that mRNA stabilization is associated with p180 expression has generated a testable hypothesis as to how increases in secretory pathway proteins could occur in the terminal differentiation in a variety of tissues including pancreas, liver, mammary gland, and plasma cells of the immune system.

Materials and methods

Yeast strains

S. cerevisiae strain SEY6210 (MAT α , *leu2-3*, *ura3-52*, *his3- Δ 200*, *trp1- Δ 901*, *lys2-801*, *suc2- Δ 9*) (Wilsbach and Payne, 1993) was used in the experiments involving *SEC61-lacZ* and membrane fractionation. Strain BY4733 (MAT α , *his3 Δ 200*, *leu2 Δ 0*, *met15 Δ 0*, *trp1 Δ 63*, *ura3 Δ 0*) (Brachmann et al., 1998) was used for the generation of the *tet-SEC61* and *tet-GFP* strains. *Aire1* was created in the strain FY1679-18B (MAT α , *ura3-52*, *trp1- Δ 63*, *leu2 Δ 1*, *his3 Δ 200*, *GAL2*) (Winston et al., 1995) using PCR-based gene replacement. Primers consisting of 40 bases up and downstream of the *IRE1* coding sequence and 20 bases identical to sequences in pRS303 were used to amplify the *HIS3* gene. (5'-ATGCGTCTACTTC-GAAGAAACATGTTAGTATTGACACTGCgattgtactgagagtgcacc-3' and 5'-TTATGAATACAAAAATTCACGTAAAAATTTGATCGTCACTTcatctgtgcgtcacccg-3'). Transformants were selected on media lacking histidine, confirmed to be devoid of *IRE1* by PCR analysis, and individual colonies were tested for sensitivity to tunicamycin. The tet-repressible *SEC61* strain was created by replacement of the region between *CSR1* gene and the *SEC61* gene with DNA between the *CSR1* EcoRV site and *SEC61* BamHI site from the pCM182 tet-*SEC61*. Gene replacements were confirmed by PCR.

Growth conditions and yeast transformations

Yeast cells were grown in 4% dextrose, 0.17% Difco yeast nitrogen base without amino acids and ammonium sulfate, and 5% ammonium sulfate (all Fisher Scientific) with the addition of nucleotides and amino acids as appropriate. Transformations were performed using lithium acetate transformation procedures as described (Gietz et al., 1995). Induction of expression of p180 or its derivatives from pYEX-BX plasmids was performed by growing the cells overnight to an OD of 0.5–3.5, diluting the cells to an OD of 0.5, and adding of CuSO₄ to 0.5 mM. Cells were induced for 5–7 h.

Where indicated, tunicamycin (Calbiochem) was added to 2 $\mu\text{g}/\text{ml}$ for 2 h and doxycycline (Sigma-Aldrich) was added to 5 $\mu\text{g}/\text{ml}$.

Plasmids

Plasmids were constructed according to standard techniques. The p180 constructs (full-length, ΔCT , ΔNT , MA) in pYEX-BX have been described previously (Becker et al., 1999). pBSK-TRP1 was a gift from Greg Payne (University of California at Los Angeles, Los Angeles, CA). The SEC61-LacZ plasmid encodes DNA from -1,000 to +51 of the SEC61 gene, fused to the *E. coli* LacZ gene in the XhoI-XbaI sites of pRS314 (Sikorski and Hieter, 1989). pMCZ-Y was a gift from K. Mori (HSP Research Institute, Shimogyoku, Japan) (Mori et al., 1996). pCM182 was a gift from E. Herrero (Universitat de Lleida, Spain) (Gari et al., 1997). The tet-SEC61 plasmid was created by PCR amplification of a 3' truncation of the CSR1 gene followed by digestion with EcoRV (endogenous) and BglI (primer-encoded) and a BamHI-EcoRI digest of pBSK-TRP1 followed by simultaneous ligation of these DNA fragments into the EcoRI-EcoRV sites of pCM182. Amplification of the coding sequence for SEC61 was followed by digestion with BamHI (in primer) and StuI (endogenous) and cloning of this fragment into the same sites in pCM182.

Transcription run-on assay

Transcription run-ons were performed essentially as described (Parker et al., 1991), except that ATP was added to 6 mM and phosphocreatine and creatine phosphokinase were omitted. PCR products encoding the entire open reading frames of the SEC61, PYK1, and GFP genes and from nucleotides 1200–2048 of the KAR2 gene were gel purified using a Gel Extraction Kit (Qiagen), precipitated, and redissolved in ddH₂O. PCR products were denatured by the addition of NaOH to 125 mM and 1 μg of DNA was spotted on to Magna Membranes (Osmonics, Inc.). Radiolabeled transcripts were isolated as the supernatants from the protein precipitation step of the MasterPure genomic DNA isolation kit (Epicentre), and incubated overnight at 42 degrees with blots that had prehybridized in prehybridization/hybridization solution (Brown and MacKay, 1997). Blots were rinsed twice, washed twice for 10 min at 42 degrees and twice for 30 min at 50 degrees in 0.1 \times SSC, 0.5% SDS and exposed PhosphorImaging screens.

Membrane fractionation

Strains harboring the YEX vector alone or the ΔCT construct of p180 were induced in wild-type yeast for 6 h with copper sulfate. Approximately 1,000 OD₆₀₀ of each strain was harvested by centrifugation. The cells were ground with a mortar and pestle in liquid nitrogen and resuspended in 5 ml Buffer I (20 mM Hepes, pH 7.4, 100 mM potassium acetate, 2 mM magnesium acetate). The fractionation was performed as described (Stoltenberg et al., 1995), except RNA from the pellet containing the membrane-bound polysomes was released by extraction with glass beads in LETS buffer (0.1 M LiCl, 0.01 M EDTA, 0.01 M Tris/HCl, pH 7.4, 0.2% SDS) instead of 0.2% sodium deoxycholate and 0.5% Tween-20. RNA was extracted as described below.

RNA isolation and Northern blot analysis

For total cellular RNA extraction, RNA was isolated as a by-product of DNA using the MasterPure genomic DNA isolation kit (Epicentre) and dissolved in formamide. For membrane fractionation experiments, RNA was extracted several times from free and membrane-bound polysome suspensions in an equal volume of DEPC H₂O-saturated phenol/chloroform/isomylalcohol (49:49:2), precipitated overnight at -20°C in isopropanol, and dissolved in formaldehyde. Five micrograms of RNA were loaded per lane for Northern blots, except for tet-SEC61 and tet-KAR2ssGFP blots, where 3 μg RNA were used. Gel electrophoresis, capillary transfer to Magna Membranes (Osmonics) and probing and washing of the blots were performed as described (Brown and MacKay, 1997). DNA fragments used as probes were generated from yeast genomic DNA or plasmid DNA with the following oligonucleotide primers: KAR2, 5'-AACTGCAGATGTTTTCAACAGACTAAGCGC-3' and 5'-ACGCATGTGCGACCTACAATTCGTCGTGTTCCGAA-3'; SEC61, 5'-CTAGCTGTCGACATGTCTCCAAACCGTGTCTAGACT-3' and 5'-AACTGCAGTCACATCAAAATCAGAAAACTCTGGAACG-3'; INO1, 5'-CCTTGATTATTGTTTC-3' and 5'-ATCTCTCTTGAATCTAGTTGG-3'; PGK1, 5'-AACGTCCATTGGACG-GTAA-3' and 5'-TCTGTGACGAACCTTGGCA-3'; PYK1, 5'-CTAGCTGTCGAGATGTCTAGATTAGAAAGATTGACCTCATTAAC-3' and 5'-AACTGCAGTTAAACGGTAGAGACTTGCAGAAAGTGTG-3'; GFP, 5'-GGGGATCCCATGCTCGAGAGTAAAGGAGAAGAAC-3' and 5'-GTGTTTGTATAGTTCATCCATGCCATG-3'; PEP4, 5'-GCATTATGCCATTGGCCTT-3' and 5'-GTGTTTGTGAGAAATGAACCTTCCA-3'. Radiolabeling was performed using the Random Prime DNA labeling kit (Invitrogen

Life Technologies, Inc.) according to the manufacturer's instructions. Blots were exposed to PhosphorImaging screens, quantitated using ImageQuant software (Molecular Dynamics). Values reported reflect normalization to levels of PYK1 or PGK1 RNA, except for membrane fractionation experiments, where RNA levels were normalized to scanned and quantified levels methylene blue-stained RNA.

β -galactosidase assays

Quantitation of β -galactosidase activity was performed using liquid assays with *o*-nitrophenyl- β -D-galactopyranoside (ONPG) as a substrate. Yeast transformants were grown overnight in appropriate synthetic media, then diluted to 0.5 OD, and grown for 5 h with 0.5 mM CuSO₄ to induce expression of p180 constructs. 10 ml cells were harvested, extracts prepared, and enzyme activity quantitated as described (Breedon and Nasmyth, 1987) with the following modifications: cells were lysed in Z-buffer without β -mercaptoethanol and reactions were performed in a microtiter plate with 5 μl extract, 100 μl Z buffer with 1 mM DTT in place of β -mercaptoethanol, and 0.7 mg/ml ONPG. Change in absorbance at 430 nm was measured following addition of 30 μl 1 M Na₂CO₃ to stop the reaction. Protein concentration was measured with the Bio-Rad Laboratories protein assay according to the manufacturer's instructions. Units are defined as $1000 \times A_{430}/(c \times t \times v)$ where *c*, protein concentration in mg/ml; *t*, time of reaction; and *v*, volume of extract in μl .

Electron microscopy

Yeast cells were grown to mid-log phase in the presence of copper to induce expression of p180 constructs. Approximately 10⁸ cells were harvested, washed once in 100 mM Tris-sulfate, pH 9.0, once in 100 mM Tris-sulfate, pH 9.0 + 10 mM DTT, and once in sorbitol buffer (1.2 M sorbitol, 20 mM potassium phosphate, pH 7.4, 5 mM DTT). Cells were resuspended in spheroplasting media (SD media + 1.2 M sorbitol, 20 mM potassium phosphate, pH 7.4, 5 mM DTT) with 100 U oxalylase and incubated at 45°C for 30 min. Cells were fixed in two steps: one 30 min incubation at 4°C in 4% glutaraldehyde solution (4% glutaraldehyde, 0.1 M cacodylic acid, 1.2 M sorbitol, 20 mM potassium phosphate, pH 7.4), and one 20 min incubation at 4°C in 2% glutaraldehyde solution (0.375 M sodium cacodylate, 3.75% sucrose, 2% glutaraldehyde). Cells were washed three times in 0.5 M sodium cacodylate, 5% sucrose, and resuspended in 2% glutaraldehyde solution. The remainder of the process was performed as described (Block-Alper et al., 2002).

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